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Expression profile of Let-7s in peripheral blood mononuclear cells of normal and severe preeclampsia pregnant women

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ABSTRACT

Headings aim: We aimed to investigate if the let-7 s expression level in the serum of peripheral blood from pregnant women with severe pre-eclampsia and normal pregnant women is related to the incidence of severe pre-eclampsia.

Methods: Total RNA was extracted from collected peripheral blood mononuclear cells from 20 or over weeks pregnant women diagnosed with severe pre-eclampsia (age: 31.57 ± 4.94) and normal pregnant women (age: 29.75 ± 4.6) respectively, followed by real-time PCR to examine the expression of let-7 s. Correlation between let-7 s expression level and maternal age or body mass index of the normal pregnant women were also analyzed using SPSS21.0 software.

Results: Let-7a and let-7 g were significantly increased in pregnant women with severe pre-eclampsia by 4.67 fold and 2.37 fold respectively compared to the normal pregnant women, whereas there was no significant difference in let-7b and let-7i. Moreover, there was no correlation between maternal age or body mass index and the expression level of let-7a, let-7b, let-7 g, and let-7i.

Conclusions: In conclusion, let-7a and let-7 g were significantly increased in the PBMCs of severe pre-eclampsia women compared to normal controls. Moreover, their expression level was not correlated to the maternal age or body mass of patients. Our data indicated that let-7a and let-7 g may be considered as predictive markers for SPE.

1. Introduction

Preeclampsia (PE) usually occurs in pregnant women post 20 weeks gestation and is considered as a serious pregnancy problem for its incidence rate at 3–6% around worldwide.(Myatt and Roberts, 2015) Currently PE is considered as a syndrome, not a disease because of its complexity and multiple symptoms own to the involvement of several systems such as vascular, rental, coagulation, and liver.(Myatt and Roberts, 2015) Traditionally, PE is defined by hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) and proteinuria (protein > 300 mg/24 h).(Mol et al., 2016; Lambert et al., 2014) With the increasing clinical data, PE could be diagnosed in the absence of proteinuria according to the new guideline of American College of Obstetricians and Gynecologists (ACOG) in 2013(American College of Obstetricians and Gynecologists, 2013). PE greatly affects maternal and perinatal morbidity and mortality for the lack of safe and effective therapeutic strategies, which accounts for > 75,000 maternal deaths yearly in developing countries. Severe preeclampsia (SPE) is a more serious disorder of pregnancy, which causes more harms to pregnant women and fetal, even later in life.(Myatt and Roberts, 2015; Mol et al., 2016; Lambert et al., 2014; American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy, 2013; Duley, 2009).

Increasing evidence demonstrates that pregnant women with higher maternal age, pre-gestational or gestational diabetes, chronic hypertension, and low socioeconomic condition have a higher incidence rate of PE.(Caritis et al., 1998; Kaaja, 2008) These factors could cause oxidative stress, inflammatory, abnormal invasion of trophoblast, shallow placental implantation, abnormal immune between maternal and fetus and aberrant gene expression to induce the pathogenesis of PE (Jauniaux et al., 2006; Roberts and Cooper, 2001; Redman and Sargent, 2003; Redman and Sargent, 2010). To understand the molecular mechanism of the pathogenesis of PE will not only enrich our basic

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knowledge in this area, and also provide potential therapeutic targets in clinical practice. Recently, studies focusing on the role of microRNAs (miRNAs) in human disease development become a hot research area. MiRNAs are a family of endogenous and small non-coding regulatory RNAs and have been reported to be involved in diverse biological processes including cell differentiation, apoptosis, and development through mRNA degradation or translational inhibition mechanism. (Bartel, 2004; Inui et al., 2010) To date, several studies have been conducted to investigate miRNAs' potential roles in the pathogenesis of PE, and aberrant expression of some miRNAs, such as miR-210, miR-181a, miR-15, miR-515, let-7, miR-223, and miR-126/126*, have been found in placental tissue or antenatal circulatory samples from PE patients, indicating miRNAs may play fundamental roles in a variety of physiological and pathological processes during the pathogenesis of PE. (Winger et al., 2015; Winger et al., 2014; Sheikh et al., 2016; Xu et al., 2014) Among of these discovered miRNAs, the let-7 family attracted our special attention because of their widely documented functions in biological processes such as cancer cell invasion and metastasis, embryonic development as well as cell proliferation, differentiation, and apoptosis.(Roush and Slack, 2008; Su et al., 2012; Boyerinas et al., 2010; Chen et al., 2013; Zhao and Popel, 2015; Wang et al., 2011; Hau et al., 2012) A number of studies reported that overexpression of let-7 suppressed the growth and invasion ability of cancer cells.(Hau et al., 2012; Qian et al., 2011; Yan et al., 2015; Kim et al., 2012; Lee et al., 2011; Wang et al., 2012) In addition, some evidence indicated that the behaviors of trophoblasts in cell proliferation and invasion are highly similar to that of cancer cells.(Soundararajan and Rao, 2004) Thus we hypothesized that let-7 may be involved in the process of trophoblast invasion into the maternal uterus which is a vital stage in the establishment of pregnancy.

Currently, fewer studies about Let-7 expression in PE have been reported. A study led by Noack et al.(Noack et al., 2011) presented the reduction of let-7b in the placenta of 5 pregnancy woman with PE compared to one normal pregnant woman. Yang and coworkers found that the expression of let-7a and let-7f-1 was increased in the serum of 4 pregnancy woman with PE compared to one normal pregnant woman, while deduction of let-7d and let-7 f. (Yang et al., 2011) Xu et al.(Xu et al., 2013) reported that lower expression of let-7i in placenta tissues was detected in pregnant women with SPE compared to normal pregnant women. Though studies discovered the aberrant expression of Let-7 member in PE patients, it is still hard to clarify their expression pattern because of limited participants and various sources of total RNA.

Whether Let-7 s could be used as predictive biomarkers in clinical diagnosis remains unclear, and more investigations are needed to obtain a consistent and robust let-7 s family expression pattern. In this presentation, we investigated the expression level of let-7a, let-7b, let-7 g, and let-7i in the serum of peripheral blood from 27 pregnant women with severe preeclampsia and 27 normal pregnant women. The purpose of this project is to explore the relationship between let-7 s level and SPE and to provide a scientific basis for clinical practice in the screening test and diagnosis for PE patients.

2. Materials and methods

2.1. Guideline for participant recruitment

Recruited participants were selected from patients diagnosed with SPE or normal pregnancy after 20 weeks' gestation according to the diagnostic standards of Obstetrics (Xie X and Gou L, 8th version (Xie and Gou, 2013). All procedures were approved by the human research ethical committee of the Second Hospital of Jilin University. These patients routinely visited the obstetrics department of the second hospital of Jilin University from October 2015 to February 2016. Briefly, the inclusion criteria for pregnancy with SPE are featured with hypertension (systolic pressure ≥ 160 mmHg and (or) diastolic pressure ≥ 110 mmHg) and proteinuria (> 2.0 g/24 h or persistent proteinuria (+ +) and over). The inclusion criteria for normal pregnancy without PE (control group) are featured with normal pregnancy process, normal blood pressure (under 140/90 mmHg) and no proteinuria. All participants are natural and singleton pregnancy and are not under the process of delivery. Each patient was informed of the use of blood samples and signed informed consent. Pregnant woman with cardiovascular disease, liver and kidney disease, diabetes, acute and chronic infectious diseases, and other complications of pregnancy were excluded. Prior to the recruitment, participants were also informed of the aims of this study and the use of collected blood as well as the rights of each party. Based on the patients' hospital record and above recruiting criteria, 27 pregnant women with SPE and 27 normal pregnant women as control were chosen in our study. Their clinical information was listed in Table 1.

Table 1
Characteristics of the study population ($N = 54$).

Maternal characteristics	SPE Group	Normal control group	P value
	($n = 27$)	($n = 27$)	
Age (year)	31.57 ± 4.94	29.75 ± 4.6	$P > .05$
Gestation week (weeks)	34.98 ± 3.09	36.4 ± 1.94	$P > .05$
Systolic pressure (mmHg)	168.7 ± 18.3	123.8 ± 11.5	$P < .05$
Diastolic pressure (mmHg)	109.5 ± 11.6	77.8 ± 9.8	$P < .05$
Proteinuria (+)	2.6 ± 0.9	(−)	

$P < .05$: significant.

2.2. miRNA isolation from PBMCs

Venous blood samples were drawn from 20 or over weeks pregnant women diagnosed with SPE and normal pregnant woman respectively, followed immediately by isolation of peripheral blood mononuclear cells (PBMCs). To isolate PBMCs, whole blood was first diluted with PBS (equal volume to the sample; 1:1), and was gently layered over an equal volume of Ficoll in a Falcon tube, followed by the centrifugation at 400*g* for 30 min at room temperature until the visibility of separation layer. PBMCs were carefully and gently removed from the second layer using a Pasteur pipette and added to warm medium. Then PBMCs were centrifuged at 200*g* for 10 min at room temperature to remove the resulting supernatant. Collected PBMCs were immediately used for total miRNA isolation using miRcute miRNA isolation kit per manufacturer protocol (TIANGEN Biotech, China). Briefly, PBMCs were first lysed in MZ lysis buffer. Obtained lysate supernatant was purified with chloroform and precipitated by using ethanol, followed further purification with the miRspin column. The concentration and purification were determined using Ultraviolet Spectrometer.

2.3. Reverse transcription

Reverse transcription was conducted by employing miRcute miRNA First-Strand cDNA Synthesis Kit according to manufacturer protocol (TIANGEN Biotech, China). Briefly, 1.0 μg total RNA was added to reverse transcription system in the final volume of 20 ul with the presence of polymerase, rATP, reaction buffer, primers, quant RTase, RNsin, and super pure dNTPs. Reverse transcription reaction lasts for 60 mins at 37 °C. Reverse transcription product was used for real-time PCR to investigate miRNA expression.

2.4. Real-time PCR

QPCR was performed using miRcute miRNA qPCR Detection Kit (TIANGEN Biotech, China) with the presence of 0.5 μl of cDNA template, SYBR Green Supermix, and primers (Let-7 specific primers listed in Table 2, universal primer and U6 primer were provided by company)

Table 2
Primers list.

Gene	Forward primer sequence
hsa-let-7a	5'-GCG GTG AGG TAG TAG GTT GTA TAG-3′
has-let-7b	5′-GGT GAG GTA GTA GGT TGT GTG GTT-3′
has-let-7 g	5'-GCG GTG AGG TAG TAG TTT GTA CAG-3′
has-let-7i	5'-CGG TGA GGT AGT AGT TTG TGC TG-3'

in 20 ul reaction mixture. The reaction was performed using the Light Cycler Real-Time system (Roche Company). Thermal cycling was under conditions: 2 mins at 94 °C, followed by 40 cycles of 94 °C for 20 s and 60 °C for 34 s. Expression fold change will be calculated with the standard $2^{-\Delta\Delta Ct}$ formula. U6 is used as an internal loading control.

2.5. Statistics analysis

The normal distribution of data from maternal age and BMI of normal women population was confirmed using SPSS21.0 software, and used them for the analysis of Pearson correlation coefficient (R) with lets expression level, followed by the verification using Spearman. The normal distribution of data from the normal and patient population was also confirmed using SPSS21.0, followed by the independent *t*-test for significance. We defined $R \leq 0.3$ as low correlation, $0.3 < R \leq 0.6$ as moderate correlation, $0.6 < R \leq 0.8$ as high correlation. Student's t-test was conducted in comparing the let-7 s expression in peripheral blood mononuclear cells from SPE and normal pregnant women. Results presented as mean ± standard deviation (S.D). *P*-value < .05 will be considered statistically significant.

3. Results

Expression of let-7a, let-7b, let-7 g, and let-7i in peripheral white blood cells.

As Fig. 1.A shown, the expression of let-7a, let-7b, let-7 g, and let-7i was able to be detected using real-time PCR reaction method, and exhibited different expression level in normal pregnant women. Among these miRNAs, Let-7a has the highest expression level compared to that of let-7b, let-7 g, and let-7i, and the later three miRNAs have a similar expression level (Fig. 1.B). When comparing to the normal pregnancy women without PE, let-7a and let-7 g were significantly increased in pregnant women with SPE by 4.67 fold and 2.37 fold respectively, whereas there is no significant difference in let-7b and let-7i (Fig. 1.C). These results indicated that upregulated let-7a and let-7 g may be involved in the pathogenesis of SPE.

Correlation analysis between maternal age or body mass index and the expression level of let-7a, let-7b, let-7 g, and let-7i.

Maternal age and body mass index have been reported as two important factors involved in the incidence of PE.(Bodnar et al., 2005; Duckitt and Harrington, 2005; Lamminpää et al., 2012) These two factors of the participants recruited in our study were various. Therefore, to determine if these factors have effects on the expression of let-7a, let-7b, let-7 g, and let-7i, we investigated the correlations between maternal age or body mass index and let-7 expression level in normal pregnant women without PE. As shown in Figs. 2 and 3, there was no significant correlation between maternal age or body mass index and the expression level of let-7a, let-7b, let-7 g, or let-7i in peripheral white blood cells ($P > .05$) (Tables 3 and 4). These data indicated that maternal age and body mass index did not influence the expression of let-7a, let-7b, let-7 g, or let-7i.

4. Discussion

As mentioned above, PE affects maternal and perinatal morbidity and mortality in clinical practice for the lack of specific predictive markers and effective therapy. Early diagnosis and effective therapeutic strategies are in great need. A study led by Winger et al. indicated that Let-7 family could be used as predictive markers in early pregnancy stay by detecting circulating miRNA level.(Winger et al., 2015; Winger et al., 2014) In this study, we for the first time in the world investigated the expression of let-7a, let-7b, let-7 g, and let-7i in peripheral white blood cells from pregnant women with SPE and normal pregnant women without PE.

Our research found that let-7a was increased in the serum of pregnant women with SPE, which is consistent with the study led by Yang and coworkers.(Yang et al., 2011) On the other hand, there is no significant difference in let-7b and let-7i in the serum of pregnant women. However, Noack et al.(Noack et al., 2011) presented the reduction of let-7b in the placenta of 5 pregnant women with PE compared to one normal pregnant woman. And Xu et al.(Xu et al., 2013) reported that lower expression of let-7i in placenta tissues was detected in 20 pregnant women with SPE compared to 20 normal pregnant women. This difference might be caused by the sources of materials and the employed methods. We also first time presented the increased expression

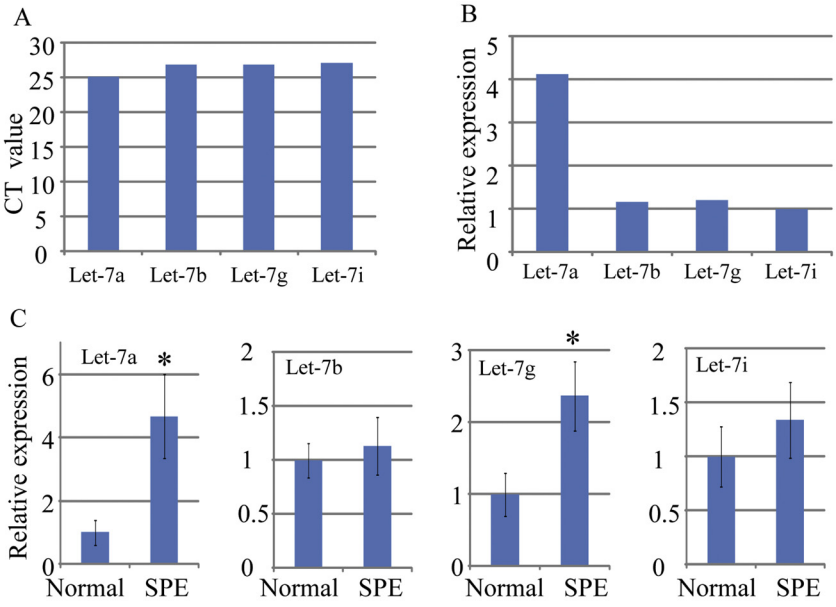

Fig. 1. Detection of let-7 expression in PBMCs of SPE and normal pregnant women. A: QPCR CT value of let-7a, let-7b, let-7 g, and let-7i in PBMCs of normal pregnant women. B: Relative expression of let-7a, let-7b, let-7 g, and let-7i in PBMCs of normal pregnant women, expression of let-7i was set as 1. C: Relative expression of let-7a, let-7b, let-7 g, and let-7i in PBMCs of SPE and normal pregnant women, expression of each let-7 in normal pregnant women was set as 1. $p < .05$ was considered statistically significant (*).

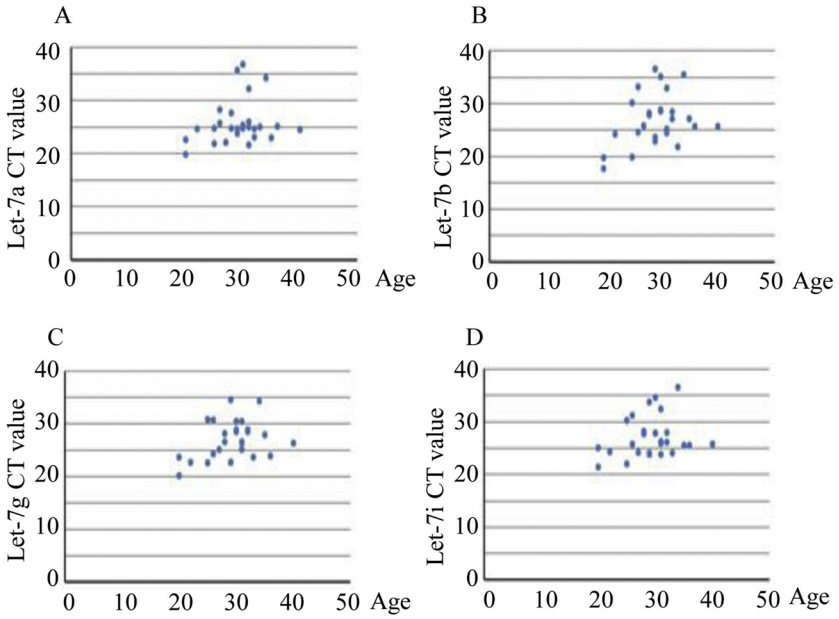

Fig. 2. Scatter dot plot of CT value of the let-7 expression in white cells from normal pregnant women of different age. A: let-7a; B: let-7b; C: let-7 g; D: let-7i.

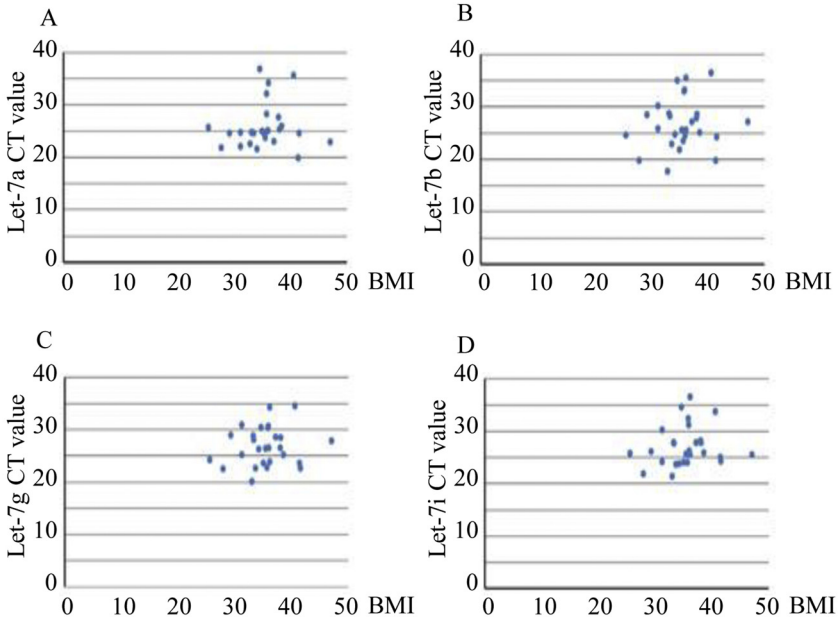

Fig. 3. Scatter dot plot of CT value of let-7expression in white cells from normal pregnant women of different BMI. A: let-7a; B: let-7b; C: let-7 g; D: let-7i.

Table 3
Correlation between age and let-7 expression.

Let-7	R value	P value
Let-7a	0.333	0.097
Let-7b	0.289	0.152
Let-7 g	0.3	0.137
Let-7i	0.18	0.379

$P < .05$: significant.

Table 4
Correlation between BMI and let-7 expression.

Let-7	R value	P value
Let-7a	0.106	0.598
Let-7b	0.148	0.46
Let-7 g	0.155	0.44
Let-7i	0.159	0.428

$P < .05$: significant.

level of let-7 g in SPE patients. Moreover, we did not detect that the expression of let-7a, let-7b, let-7 g, or let-7i was correlated with the maternal age or body mass index, indicating that let-7a and let-7 g might be used as independents predictive markers in the clinical practice.

A number of studies demonstrated that let-7a and let-7 g function as a tumor suppressor through inhibiting the invasion and metastasis ability of cancer cells.(Qian et al., 2011; Kim et al., 2012) Trophoblast cells share similar characteristics with cancer cells in development and invasion behaviors.(Soundararajan and Rao, 2004) Thus, higher expression of let-7a and let-7 g in the serum of pregnant women might affect the invasion of trophoblast into the maternal decidua and lack of

spiral artery remodeling, causing the abnormal development and maintenance of placenta. It is widely believed that shallow trophoblast invasion into the maternal decidua and lack of spiral artery remodeling, (Meekins et al., 1994) resulting in maintenance of high-resistance blood vessels, lead to uteroplacental hypoxia in preeclampsia. In addition, let-7a and let-7 g have been reported as hypoxia-inducible miRNAs,(Chen et al., 2013; Kulshreshtha et al., 2008) and moreover, increasing evidence indicated that hypoxia occurs during the process of PE.(Lunell et al., 1982; Zamudio et al., 2007; Soleymanlou et al., 2005) In all, this evidence indicated that let-7a and let-7 g may in part play a vital role in the pathogenesis of PE and might be used as predictive markers for the screening test of pregnant women throughout the pregnancy. Our future study will focus on the study of the target genes of let-7a and let-7 g as well as the involved pathways to provide further insight of the pathogenesis of PE and potential therapeutic approaches for PE patients in clinical practice.

Disclosure

The authors declare that there is no conflict of interest regarding the publication of this article.

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